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TITLE: Development of an Assay for Prostate Cancer Based on Methylation Status of Glutathione S-Transferase (p)

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13. ABSTRACT (Maximum 200 Words)

Gluthiaone-s-transferase (GST)-π gene is frequently methylated in prostate cancer and recent evidence has linked aberrant expression of this gene with the development of prostatic adenocarcinomas (PACs). The main purpose of this study is to evaluate GST-π protein expression by immunohistochemistry (IHC) and develop a simple DNA amplification assay for detection of GST-π methylation status. 220 cases including 180 PACs and 20 cases each of normal and benign hyperplastic tissues were identified. Protein expression was evaluated on 4 μm paraffin sections in all cases using a polyclonal rabbit antihuman GST-π antibody by an automated method. Methylation status was assessed in 176 cases including 155 PACs and 21 benign tissues using 466/468 and 425/430 primer pairs by a gel based PCR amplification method. 175 of 180 (97%) PACs demonstrated loss of GST-π protein expression in comparison to the presence of this protein in 100% of benign tissues including normal and BPH specimens. 127 of 155 (82%) cases of PACs and 1 of 21 (5%) benign tissues demonstrated methylated GST-π promoter DNA. Given that the overwhelming majority of PACs lacked GST-π protein expression, no correlation was found with any prognostic variables. In conclusion, GST-π protein is downregulated in almost all prostate cancers and is associated with methylation of the promoter region of the gene. Further studies are warranted to evaluate the utility of GST-π methylation assay in screening and diagnosis of prostate cancer.

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Introduction

Prostate cancer is currently the most commonly diagnosed cancer in American men. Early diagnosis through accurate screening techniques will significantly contribute to the successful management and eventual eradication of this disease. Currently, serum PSA determination remains the cornerstone of prostate cancer screening. However, more accurate screening to better differentiate patients with benign disease from those with prostatic cancer will alleviate the unnecessary surgical procedures with a significant impact on patient morbidity and health care costs. Glutathione-s-transferases (GSTs) are a family of detoxification enzymes which catalyze the conjugation of a wide variety of endogenous and exogenous toxins with glutathione. Recent literature reported that methylation of the GST-π gene, which inactivates the expression of the enzyme, is a cancer specific marker in the prostate (*1*). Since the original submission of the current research project, considerable new evidence has been published linking aberrant expression of the GST-π gene with the development of prostate cancer (*2, 3*). In a recent study of prostate cancer tissues using a PCR based-assay for GST-π methylation, 24/32 (75%) cases were positive and the results correlated with disease stage (*4*). It has also now been shown that GST-π methylation is not confined to specific sites in the promoter, but occurs throughout the entire CpG island sequence (*5*). Interestingly, hypermethylation is regularly identified in the GST-π gene in normal and hyperplastic prostate tissues outside the promoter region with no loss of gene function (*5*). The main purpose of this study is to evaluate GST-π protein expression by immunohistochemistry and develop a simple quantitative DNA

amplification assay for detection of GST-π methylation status. The scope of this study is to newly develop both an automated immunohistochemistry system and a quantitative PCR based assay and apply these assays to prostate cancer tissues which constitute part of the established data base at the Albany Medical College. The ultimate goal was to detect circulating methylated GST-π DNA bearing prostate cancer cells. Additionally, application of this assay to tiny pre-operative needle biopsy specimens will significantly aid in the definitive diagnosis of prostatic carcinoma.

Body

Task 2. Assemble and blind slides and microdissected tissue samples from ≥180 prostate tumor tissues in the previously characterized Albany Medical College (AMC) collection, as well as ≥ 20 normal and ≥ BPH prostate tissue samples.

Task accomplished

180 prostatic adenocarcinomas and 20 cases each of normal and benign prostatic hyperplastic tissues were identified from the established database at the Albany Medical College. H&E slides in each case were reviewed to confirm the diagnosis. The tumors were graded (Gleason grade) and staged (AJCC). Tumors were classified as high grade when the Gleason score was 7 and above and as low grade when the score was 6 and below. Serum PSA levels and clinical follow-up were obtained from the patients' records. A post-surgical elevation of PSA level from a baseline of 0 ng/ml to >0.4 ng/ml was considered as disease recurrence. Tissue from 20 patients who underwent transurethral resection for a clinical diagnosis of benign prostatic hyperplasia were included. The 20 cases of unremarkable prostatic tissue were acquired from autopsy material on patients who died of unrelated causes. Approximately 200 μm of paraffin-embedded tissue from each case was microdissected and placed in Eppendorf tubes for gDNA isolation and PCR amplification. Unstained 4 μm sections were prepared in all cases and utilized for immunohistochemical analysis.

Task 3: Convert the methylation-specific PCR for GST-π from gel analysis to homogeneous fluorescence detection.

Task modified/completed

Our modification was to convert the methylation-specific PCR from gel to "Taqman" quantitative analysis. Taqman probes have the intrinsic advantage of discriminating against primer dimer products, improving the signal to noise ratio, but there are only a limited number of compatible probes possible in the methylation region. As an initial test we designed a "Taqman" probe for use with the Applied Biosystems Real-time Analysis Kit and our ABI 7700 Sequence Detector. We were able to obtain a good signal for methylated DNA from the LNCaP clone FGC prostate cancer cell line compared to a negative signal for control DNA from the DU145 cell line (known not to be methylated), though clearly the system is not yet optimal. We were also able to detect methylated DNA in a model template generated by methylation of a plasmid clone of the GST-π (methyl groups were introduced by use of CG methylase from New England Biolabs) (Figure 1). Thus under conditions where we can generate a PCR product detectable on gels we can also detect a specific signal using a Taqman probe.

Task 4: Isolate gDNA from each of the ≥ 220 prostate tissue samples and test each one for GST-π methylation status using the tube based Sunrise™ GST-π methylation assay.

Task modified/177 of 220 cases completed

We analyzed 177 tissue samples using the gel-based method. DNA was extracted from paraffin embedded tissue, using the "Ex-Wax" method followed by bisulfite modification. Using the 466/468 primer pair, we tested 24 samples in an initial trial and demonstrated a 39% methylation rate in PACs versus 17% in benign tissues. Subsequently, using the 425/430 primer pair, we demonstrated a significantly increased (82%) methylation rate in PACs compared to 6% in the benign tissues, based on 153 samples.

Problems associated with accomplishing this task:

Continued improvement of methylation-specific PCR.

(A)   Although early results with a methylation-specific PCR detection system were very promising, we were dissatisfied with the level of discrimination between methylated and non-methylated DNA achieved using the primers we started out with, and with the presence of extra non-specific gel bands.

A large number of PCR primers were tested for amplification of samples of DNA which had been subjected to bisulfite modification (Figure 2). All were designed to anneal preferentially to various segments containing methylated CG sites. The efficiency of amplification varied widely from one pair of primers to another. Even more problematic was a wide variation in the ability of primer pairs to discriminate between methylated and non-methylated DNA, so that the majority of pairs gave a relatively less abundant but detectable product even from non-methylated template. Though we were not able to determine the basis of these variations, we were able to define empirically 2 pairs which performed well, providing a reasonably robust amplification with no

background from non-methylated DNA. However, we continued to evaluate an exhaustive list of additional primer pairs for maximum sensitivity and specificity and finally chose to perform the GST-π methylation analysis on the test samples using primer pair 425/430.

(B)   A second way of reducing non-specific (i.e. non-methylated) signal is to eliminate the bulk of the non-methylated GST-π template DNA by pre-digesting with methylation-sensitive restriction enzymes which have sites in the region of interest (NotI, SmaI, NgoMIV, FspI). This was not successful due to a significant fraction of digestion-resistant DNA

Reamplification by LCR:

(C)   Attempts to boost the signal from small amounts of methylated DNA by re-amplification of PCR products using internal primers were disappointing. We were able to increase sensitivity in this way by about an order of magnitude, but there was a considerable background. As an alternative method of reamplification we used the ligation chain reaction (LCR); in our experiments oligonucleotides complementary to bisulfite-treated CG-rich segments in the GST-π promoter were ligated by the established LCR method, which has found increasing application in the field of detection of genetic polymorphisms. We were able to detect a good signal using small aliquots from a PCR product generated from methylated DNA (Figure 3). In our first attempts we have not yet been able to show amplification using fluorescent

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oligonucleotides, as needed for RealTime LCR detection in the proposed final methodology. We estimate a potential increase in sensitivity of at least 100-fold can be obtained in this way. Theoretically, it should be possible to do better than that and we are optimistic about using the PCR-LCR combination to reach the sensitivities needed to detect methylated DNA in blood or prostate secretions. Another possible way of achieving re-amplification is through use of Sun-Rise primers, following the initial PCR reaction.

Task 5: Develop an immunohistochemical staining method for GST-π expression.

Task accomplished

In our attempt to develop a novel automated method for immunohistochemical analysis, we used a commercially available rabbit polyclonal antihuman GST-π antibody (Vector Laboratories, Inc., Burlingame, CA). The staining protocol was developed and optimized based on the immunoreactivity of bile duct epithelium in normal human liver tissue (positive control). This unique automated IHC method is an indirect biotin-avidin system. Immunoperoxidase procedures such as this which allow direct light microscopic visualization of antigen in tissue sections are complex and involve precise optimization of various intermediate steps to enable both sensitive and specific detection of the antigen of interest. Additionally, the unique advantage of automating a test procedure is the resultant high levels of reproducibility and validity of the results.

The final IHC protocol we developed and validated utilized the Ventana ES (Ventana Medical Systems, Inc., Tucson, AZ) automated IHC instrument. The tissue

sections were deparaffinized, rehydrated and preincubated in 3% hydrogen peroxide. No antigen retrieval step was employed for the detection of GST-π protein. Slides were positioned in slide holders within the reaction chamber of the instrument. Negative control slides were included to establish background and non-specific staining of the primary and secondary antibodies and/or detection kit reagent. Through the Ventana reagent dispense system, endogenous peroxidase activity was further blocked with 3% hydrogen peroxide. Endogenous biotin was masked by the binding of avidin. Sections were incubated with the primary antibody at a dilution of 1:150 at 42°C for 32 minutes. Subsequent incubations included biotinylated rabbit immunoglobulin secondary antibody, avidin horseradish peroxidase conjugate and diaminobenzyadine (DAB) followed by copper sulfate enhancement and finally counterstaining with hematoxylin. Slides were removed from the instrument, dehydrated, coverslipped and examined for expression of the GST-π protein using a standard light microscope.

Task 6: Test prostate tissue using an immunohistochemical staining method for GST-π expression. Review slides for location of the antibody stain and tissue pathology.

Task accomplished

GST-π protein expression data was generated on the 220 prostate tissues. Immunoreactivity for this protein was scored based on both staining intensity and distribution. The staining intensity was subjectively graded as weak, moderate or intense while the distribution in tumor cells was scored as focal (<10%), regional (11-

50%), or diffuse (>50%). The overall results in each case were characterized as either positive or negative for GST-π expression.

In benign epithelium, staining was noted in the cytoplasm of the secretory epithelial cells of prostatic acini in 100% of cases (Figure 4). Additionally, the basal cells of prostatic acini demonstrated an intense, diffuse positivity for this protein (Figure 5) in all cases. The other elements that were immunoreactive for this protein included seminal vesicles (Figure 6), nerves (Figure 7) and ganglia. While the location of staining was predominantly cytoplasmic in the epithelial cells, both nuclear and cytoplasmic staining was noted in the basal cells. In all cases, the basal cell staining was more intense as compared with the epithelial cell staining. Additionally, in no case, was epithelial cell staining observed in the absence of basal cell staining.

In prostatic carcinomas, 97% of cases demonstrated loss of GST-π expression within the tumor cells (Figure 8). In all these cases, there were admixed benign prostatic elements that were positive for this protein, serving as internal controls. The remaining five PACs were positive for GST-π (Figure 9). Foci of high grade prostatic intraepithelial neoplasia (PIN) amongst these tumors also showed absence of immunoreactivity for this protein (Figure 10). Statistical analysis revealed no correlation of loss of GST-π expression with any of the prognostic variables including grade, stage, serum PSA and disease recurrence.

Task 7: Develop an in-situ PCR version of the Sunrise™ GST-π methylation assay.

Task re-evaluated.

The initial intent was to modify the gel based GST-π methylation assay using "sunrise primers" to a slide based in-situ PCR test. However, as discussed in earlier sections, the sensitivity and specificity of PCR detection is still unsatisfactory for the development of a slide-based in-situ PCR test appeared extremely limited and of questionable reliability.

Given the ease and reproducibility of our indigenously, developed automated immunohistochemistry (IHC) system which demonstrated loss of the protein in a vast majority of PACs and correlation with methylation status of the gene, we recommend a combination of IHC and gel based PCR analysis as optimum for the evaluation of GST-π gene status in PACs.

Task 8: Test prostate tissue using the in-situ version of the Sunrise™ for GST-π methylation assay. Review slides for location of the fluorescence signal from the in situ PCR assay and tissue pathology.

Task re-evaluated. See Task 7.

Task 9: Compare all GST-π methylation and expression data gathered on the ≥ 220 prostate tissue samples with the existing information already known about those samples. Write up the results and submit them to a journal such as Cancer Research for peer-reviewed publication.

Task begun, awaiting completion.

Given the extremely high rate of GST-π gene methylation and consequent protein downregulation, no correlation was found with any of the clinicopathologic prognostic variables. However, this widely prevalent GST-π abnormality can be successfully utilized as a diagnostic test that can significantly aid in the screening and early diagnosis of prostate cancer.

Two abstracts (see appendix) have been recently presented at national meetings and published in their accompanying journals. The full length manuscript is under preparation and will be submitted for publication shortly.

Task 10: Develop a DNA isolation/bisulfite modification method for small (pg-ng) quantities of DNA in serum.

Task modified.

Despite the enhanced sensitivity of the newer serum based screening tests, prostatic needle biopsy remains a requirement to establish a definitive diagnosis of prostate cancer. While in some cases adequate amount of tumor is represented in the biopsy material resulting in an unequivocal diagnosis, in others, the amount of tumor is extremely scant, precluding a definitive diagnosis and necessitating additional biopsies. Based on our IHC results, the value of GST-π protein downregulation becomes apparent as a useful tool in the diagnosis of cancer on needle biopsies with scant tumor. In this regard, the ability to demonstrate GST-π methylation by gel based PCR will further enhance diagnostic accuracy, alleviating the need for additional biopsies in morphologically equivocal cases. Clearly, a test applicable to blood or prostate

massage samples would be of great value. We will continue to develop amplification techniques to make it practicable.

Task 11: Collect ≥ 50 pre-operative serums with known PSA values and post-operative prostate tissue histology.

Task Modified.

In continuation of response to Task 10, we collected 100 cases of prostatic needle biopsies representing varying amounts of tumor as well as the numerous mimics of carcinoma (Table 1) to evaluate the utility of both IHC and PCR analysis in rendering an unequivocal diagnosis of cancer. We will compare the routine histology with protein expression and gene methylation status to evaluate the efficacy of our newly developed assays in diagnosing cancer from minute amounts of tumor represented.

Task 12: Develop an improved sensitivity Sunrise™ GST-π methylation assay for DNA isolated from serum.

Task modified. Refer to tasks 7, 8, 10, 11.

Task 13: Test a set of ≥ 50 prospective serum samples with known PSA value and prostate tumor status using the high sensitivity version of the Sunrise™ GST-π methylation assay developed in Task 12.

Task modified. Refer to tasks 7, 8, 10, 11, 12.

Task 14:   Compare the GST-π methylation data gathered on the $\geq$ 50 pre-

operative serums with the pre-operative serum PSA value and with prostate

tissue pathology results. Write up the results and submit to a journal such as

Cancer Research for peer-reviewed publication.

Task modified. Results of Task 11 to be completed and submitted for

publication.

Discussion

The results obtained both by IHC and gel-based PCR analysis in this study unequivocally confirm the utility of GST-π protein downregulation and gene methylation in the accurate diagnosis of prostate cancer. Using our indigenously developed automated IHC assay, we demonstrated protein downregulation in a vast majority of PACs, consistent with reported findings (6,7). Given the almost universal loss of this protein and the consequent lack of correlation with clinico-pathologic prognostic variables, this assay has the potential of being an extremely important tool in the diagnostic armamentarium of prostate cancer. Interestingly, foci of high grade PIN showed protein downregulation similar to infiltrating cancer suggesting that this dysregulation occurs in the early stages of cancer development. The intense positivity of the basal cells in benign acini for the GST-π protein can be effectively used as an adjunct marker to diagnose small foci of cancer on the preoperative needle biopsies and differentiate cancers from numerous other mimics (Table 1) that confound pathologic interpretation. Needle biopsies with small foci of carcinoma are not infrequently associated with equivocal pathologic diagnosis subjecting these patients for further follow-up including repeat biopsies. The presence or absence of basal cells is an important criteria in evaluating small atypical glands for cancer. While other currently widely used basal cell stains, e.g. high molecular weight cytokeratin, can successfully highlight basal cells, GST-π offers the added advantage of being expressed in basal cells and downregulated in cancer cells as opposed to its expression in secretory cells of intermixed benign acini. To this end, we are currently

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evaluating the utility of GST-π in comparison with other basal cell markers in the interpretation of small foci of atypical glands on prostatic needle biopsies.

Previous studies demonstrated hypermethylation of the CpG sites of the promoter region of the GST-π gene (1). Using DU145 and LNCaP prostate cancer cell lines, we achieved a desired level of discrimination between non-methylated and methylated GST-π DNA with the initial set of primers utilized (466/468). However, when these primers were applied to PCR amplification of DNA extracted from human prostate cancers, GST-π gene methylation was demonstrated in approximately 39% of cases. In an attempt to increase the detection rate, we evaluated numerous additional primer pairs and eventually identified one pair (425/430) that performed well on DNA from paraffin embedded PACs. We demonstrated that 82% of all PACs were associated with GST-π gene methylation which mirrors the data obtained on IHC. Our initial attempts at further increasing the sensitivity using reamplification by ligation chain reaction and quantitation by "Taqman" real time PCR analysis yielded promising results (Figures 1,3). We are currently evaluating the sensitivity of our gel-based PCR assay using the small amounts of methylated DNA obtained from foci of cancer on prostatic needle biopsies. A combination of IHC and PCR analysis will significantly aid in the accurate and definitive interpretation of questionable foci on needle biopsies, alleviating the need for performing additional biopsies before definitive therapy can be instituted.

In our opinion, based on the data obtained from this study, an even further increase in sensitivity (another order of magnitude) would be necessary to evaluate the utility of this PCR assay in detecting methylated DNA from blood samples. An

alternative, sample such as prostatic massage specimen may likely yield higher amounts of tumor derived DNA, making it possible to detect GST-π methylation using the current PCR assay. We will be testing the latter possibility in a pilot study in the near future and if successful, will apply for additional funding to implement this project.

Key Research Accomplishments

- Assembled 180 prostatic adenocarcinomas and 20 cases each of BPH and normal prostatic tissue with all relevant clinical and pathologic data pertaining to each case from the established data base at the Albany Medical College.

- Microdissected tissue samples from all cases for gDNA isolation for the tube based GST-π methylation assay and cut unstained slides for immunohistochemical staining and subsequent analysis.

- Developed a unique, automated IHC staining protocol for GST-π protein expression, stained all cases and evaluated in detail for the presence and distribution of this protein in both benign and malignant prostatic tissues.

- Developed gel-based PCR detection method for malignant prostatic tissues using optimal primers (466/468) selected after screening a large set of candidate pairs; sensitivity augmented by PCR re-amplification.

- Analyzed 23 prostate tissues (18 carcinomas, 5 benign) for GST-π methylation using this gel-based PCR assay with 39% methylation rate in PACs versus 17% in benign tissues.

- Continued optimization for sensitivity by testing numerous additional primer sets resulting in identification of primer set 425/430.

- Analyzed 153 additional prostate tissues (137 carcinomas, 16 benign) for GST-π methylation with gel-based PCR using primer set 425/430 resulting in significantly

enhanced sensitivity and specificity i.e. 82% methylation rate in PACs versus 5% in benign tissues.

- Designed a "Taqman" probe for real-time quantitative PCR to detect methylated DNA and validated this assay using appropriate controls which included prostate cancer cell lines and cloned plasmid.

- Increased sensitivity by an order of magnitude by reamplification of PCR products using internal primers (nested PCR) but with persistent disadvantage of high background.

- Performed ligation chain reaction (LCR) as a alternative method of reamplification and detected a good signal from a PCR product generated from methylated DNA.

- Assembled a database of 100 prostatic needle biopsies with varying amounts of cancer represented to test the utility of IHC and PCR assays in aiding accurate and definitive diagnosis of prostate cancer.

Reportable Outcomes

Abstracts

Two abstracts presented at National meetings and published in the journals of the respective associations.

- Kallakury BVS, Sheehan CE, Lowry CV, Salmon SL, Anand RD, Fisher HAG, Kaufman RP, Ross JS. GST-π Protein is Downregulated in High-Grade PIN and Prostatic Adenocarcinomas (PACs): A Preliminary Analysis of Association with Methylation of Gene Promoter. Presented at the 90th Annual Meeting of the United States and Canadian Academy of Pathology, Atlanta, GA, March 3-9, 2001. Published in Modern Pathology 2001; 14:112A.

- Ross JS, Kallakury BVS, Sheehan CE, Salmon S, Anand RD, Fisher HAG, Kaufman RP, Lowry CV. Immunohistochemical Downregulation of GST-π Protein and Methylation of the Gene Promoter are Associated with Majority of Prostatic Adenocarcinomas (PACs). Presented at the 92nd Annual Meeting of the American Association for Cancer Research, New Orleans, LA, March 24-28, 2001. Published in The Proceedings of the American Association for Cancer Research 2001:42; 228.

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Presentations

- Dr. Ross presented "Prognostic Factors in Prostate Cancer" as Grand Rounds at the Massachusetts General Hospital, Boston in June 1999 highlighting the preliminary data concerning GST-π hypermethylation in prostate cancer.

- Dr. Ross discussed the GST-π hypermethylation assay at the following lectures and Visiting Professorships:

- Brigham and Women's Hospital, Boston MA, December 2000

- Albany Medical College, Albany, NY, December 2000

- Millennium Pharmaceuticals, Inc., Cambridge, MA January 2001

- University of Miami, Miami, FL, January 2001

- Dr. Ross included preliminary immunohistochemical data in the following workshop presentations:

- American Society of Clinical Pathologists National Meeting in Orlando, FL in April 1999.

- American Society of Cytopathologists National Meeting in Sacramento, CA in November 1999.

- United States and Canadian Academy of Pathology National Meeting in New Orleans, LA in March 2000.

- United States and Canadian Academy of Pathology National Meeting in Atlanta, GA in March 2001

Manuscripts

Two manuscripts as titled below are currently under preparation for submission for publication in the following journals (delayed due to change in primer set for PCR assay resulting in increased sensitivity).

- GST-π protein is downregulated in high grade PIN and prostatic adenocarcinoma and is associated with hypermethylation of the promoter region of the gene (Cancer Research).

- GST-π protein expression is downregulated in high grade PIN and prostatic carcinoma and correlates with basal cell phenotype: Comparison with basal cell markers 34β12 and CD44v6 (American Journal of Surgical Pathology).

Additional Planned Study

1.   Utility of GST-π protein expression and gene methylation in the diagnosis of prostatic carcinoma and its mimics in needle biopsy specimens.

Conclusions

In summary, we demonstrated that a vast majority of prostatic carcinomas show loss of GST-π protein and are associated with methylation of the promoter region of this gene. Hypermethylation of this site may be relatively heterogeneous in human prostate cancers as compared to prostate cancer cell lines and require modified PCR assays and reamplification strategies for enhanced sensitivity. The diffuse expression of GST-π protein in basal cells but not in neoplastic epithelial cells indicates its early role in the evolution of prostate cancer. The basal cell staining property of GST-π is of significant help in the accurate and definitive diagnosis of prostatic carcinoma on preoperative needle biopsies. The combination of GST-π protein expression and methylation status may be a powerful tool in distinguishing carcinoma from mimics on needle biopsies.

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APPENDICES

TABLE 1

Mimics of Prostatic Adenocarcinoma

I.   Hyperplastic lesions

- Atypical adenomatous hyperplasia

- Clear cell cribriform hyperplasia

- Sclerosing adenosis

- Atypical basal cell hyperplasia

II.   Atrophy related lesions

- Atrophy

- Post atrophic hyperplasia

III.   Dysplastic lesions

- High grade PIN

IV.   Normal Structures

- Cowpers glands

- Paraganglia

- Seminal Vesicle

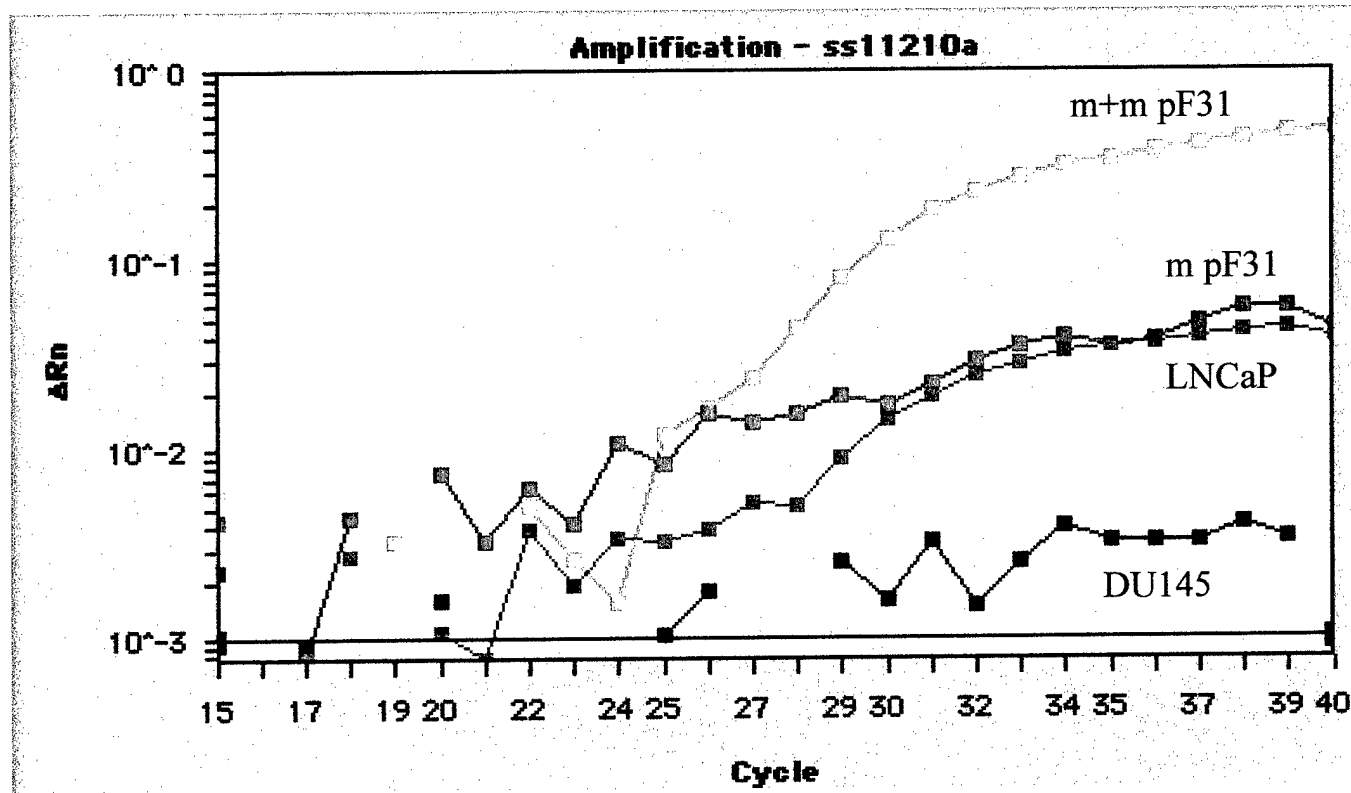**Personnel receiving pay from the research effort**

Charles Lowry, PhD

Sharon Salmon

Christine Sheehan

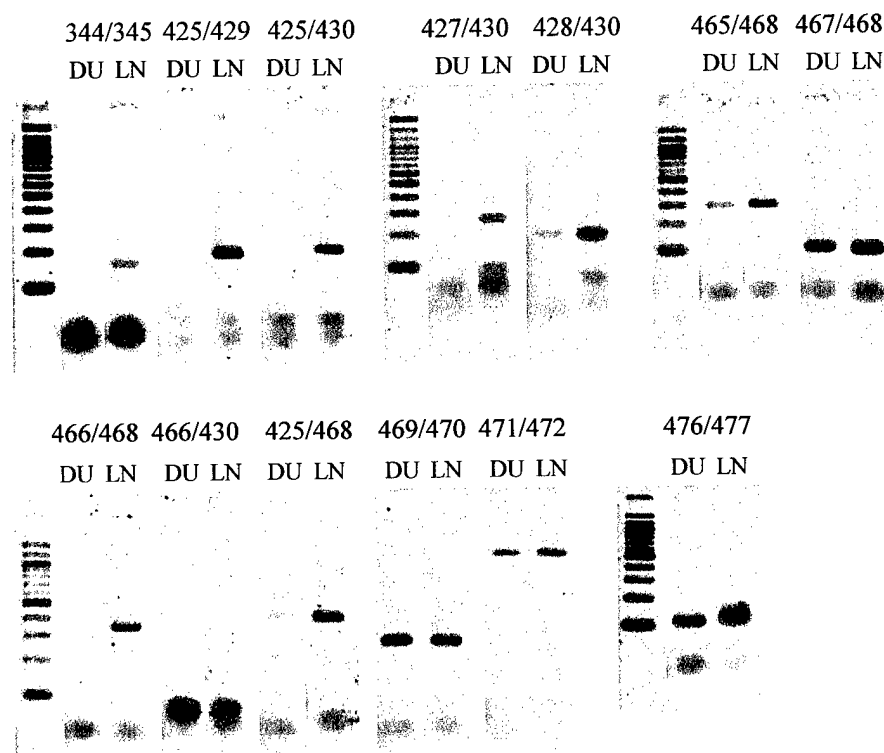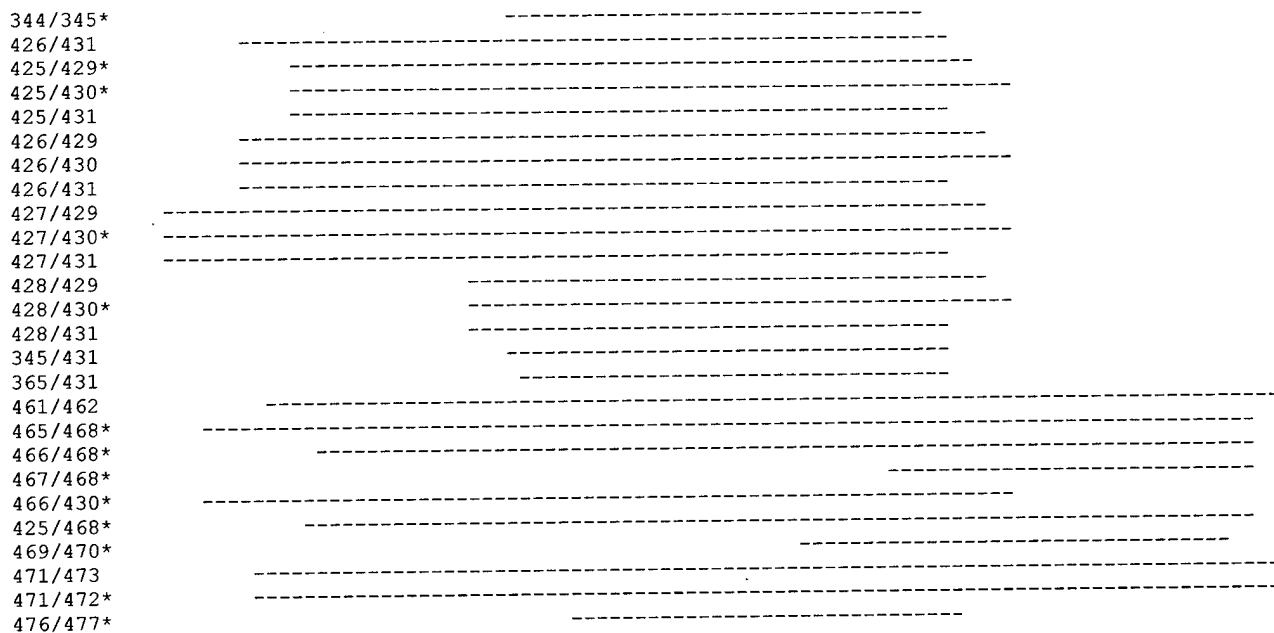Liz Paunovich (replaced Kane who replaced Reed)

Institutional "cost share" contribution toward salaries for:

Jeffrey Ross, MD

Bhaskar VS Kallakury, MD

Figure 1: Amplification of methylated GST-π sequence using a Taqman probe and ABI Prism 7700 Sequence Detector. DU145 is a human prostate cancer cell line known to have unmethylated GST-π gene, LNCaP is a human prostate cancer cell line with methylation of the GST-π gene, m pF31 is a bisulfite modified plasmid that contains the promoter region of the unmethylated GST-π gene, and m+m pF31 is a bisulfite modified and methylated plasmid that contains the promoter region of the GST-π gene.

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344/345*          ------------------------------------
426/431        ------------------------------------------
425/429*          ------------------------------------------
425/430*          --------------------------------------------
425/431        ----------------------------------------------
426/429      ------------------------------------------------
426/430      --------------------------------------------------
426/431      ----------------------------------------------
427/429    --------------------------------------------------
427/430*   ----------------------------------------------------
427/431    --------------------------------------------
428/429          ----------------------------------------
428/430*         ----------------------------------------
428/431          --------------------------------------
345/431          --------------------------------------
365/431          ------------------------------------
461/462      ------------------------------------------------------
465/468*   --------------------------------------------------------
466/468*       ----------------------------------------------------
467/468*                 ------------------------------
466/430*   ----------------------------------------
425/468*       --------------------------------------------------
469/470*                   ----------------------------------------
471/473      ----------------------------------------------------------
471/472*   --------------------------------------------------------------
476/477*         ------------------------------
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344/345 425/429 425/430   427/430 428/430   465/468 467/468
DU LN   DU LN   DU LN   DU LN   DU LN   DU LN   DU LN

466/468 466/430 425/468 469/470 471/472   476/477
DU LN   DU LN   DU LN   DU LN   DU LN   DU LN

Figure 2: Discrimination (and non-discrimination) of methylated and non-methylated GST-π DNA from tissue culture cells by PCR with various primer pairs (partial list illustrated).
DNA derived from the DU145 and LNCaP.F6C cell lines (non-methylated and methylated respectively) was bisulfite-modified and used as template for PCR reactions with various primers within the GST-π promoter . The endpoints of the PCR products for each primer pair are shown in the line drawing; the positions of methylatable CG sites are indicated by short vertical marks. Two primer pairs (425/430 and 466/468) have emerged as highly useful.
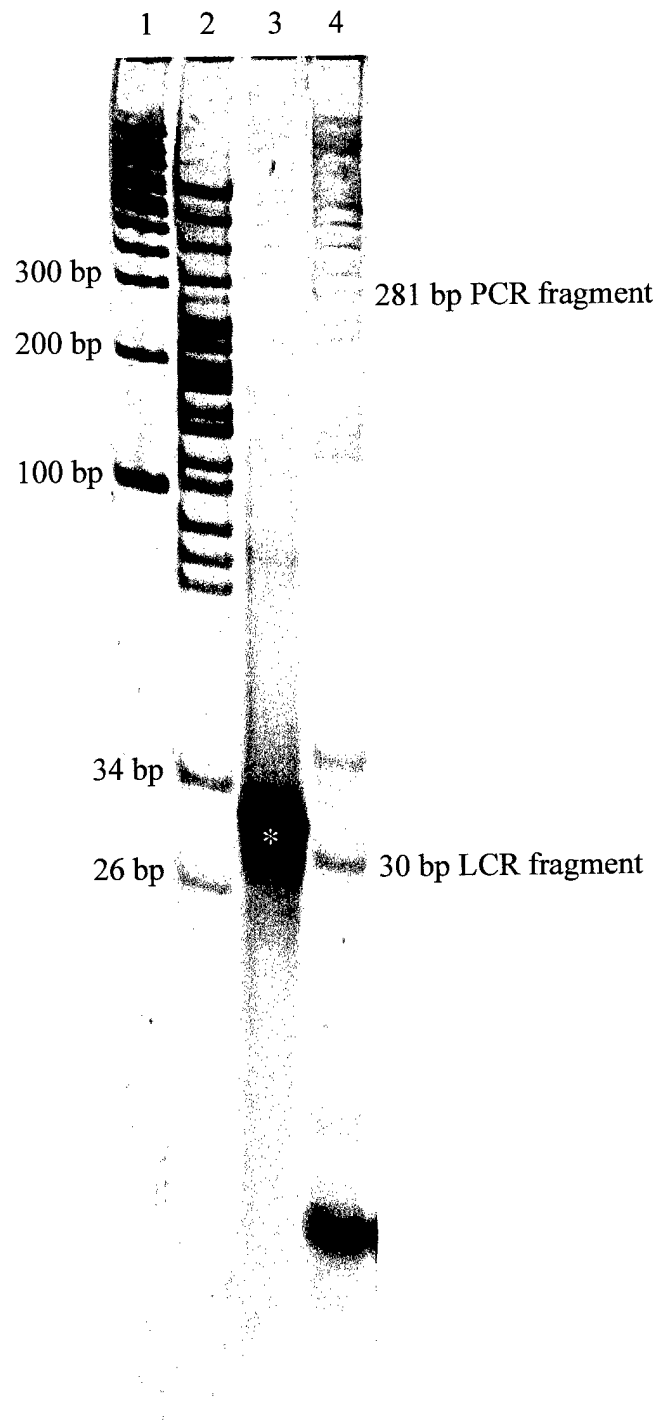
Figure 3: 12% acrylamide gel showing 30bp fragment generated in a Ligase Chain Reaction (LCR). Lane 1 - 100bp ladder; Lane 2 - pBR322 MspI digest; Lane 3 - 30bp fragment (*); Lane 4 - LCR using GST-π PCR fragment (281bp) as template to generate a 30bp LCR fragment.
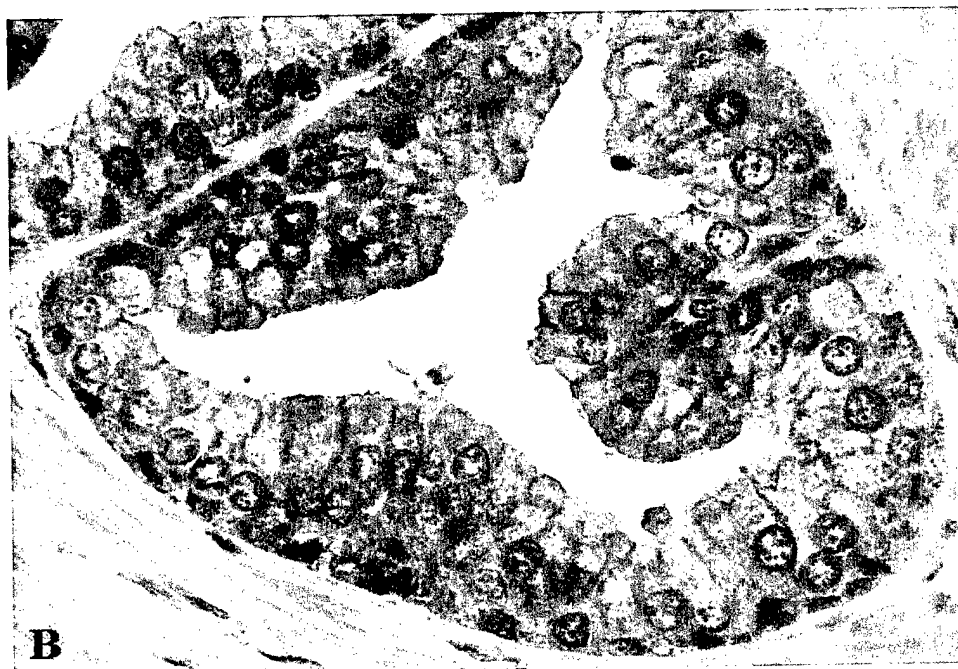
Figure 4: GST-π immunoreactivity in benign prostatic acini. Panel **A** illustrates intense, diffuse, predominately cytoplasmic positivity in the secretory epithelial cells (100X) Panel **B** shows scattered nuclear expression of GST-π protein in sporadic acini (400X). (anti-human GST–π, DAB, hematoxylin)
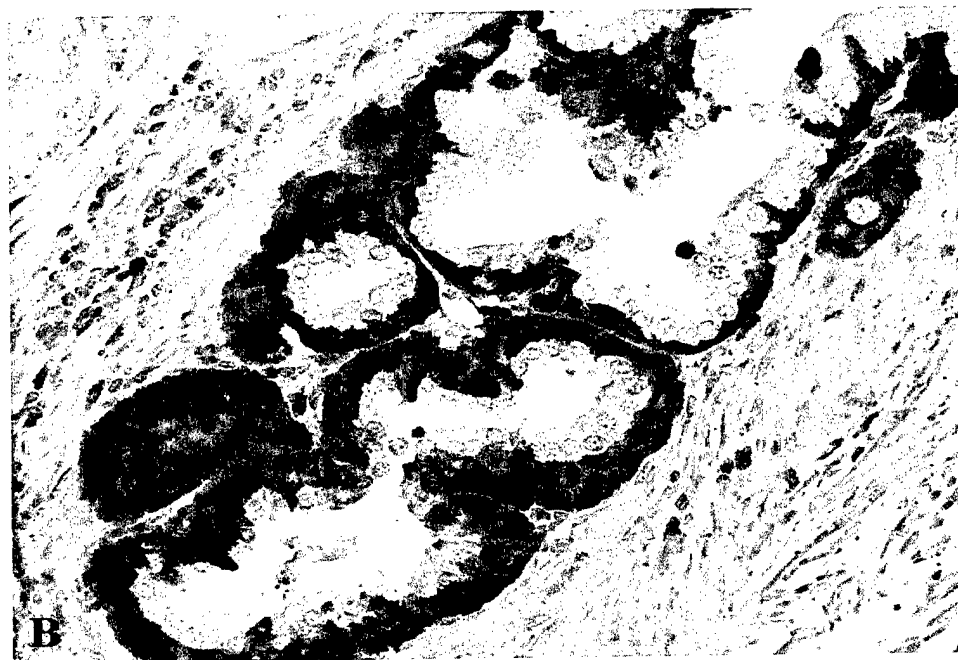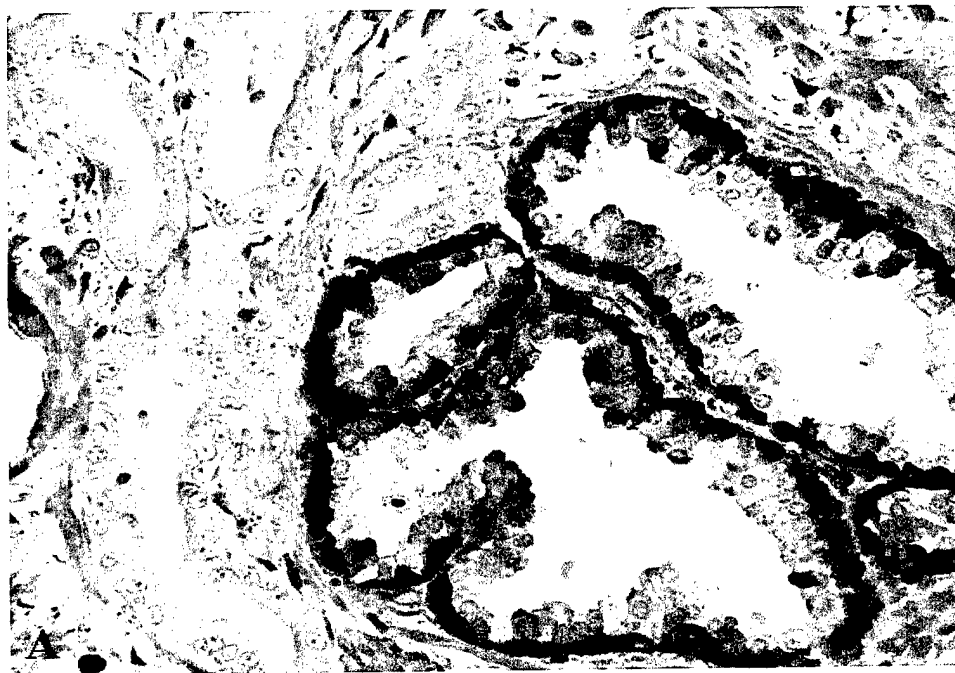
Figure 5: Panels **A & B** (200X) Intense, predominately basal cell GST-π immunoreactivity in benign prostatic acini. Panel **A** illustrates focal cytoplasmic and nuclear patterns of positivity in secretory epithelial cells. Also note in Panel **A**, a focus of low grade prostatic adenocarcinoma (Gleason score 6/10) shows loss of expression of the GST-π protein. (anti-human GST–π, DAB, hematoxylin)
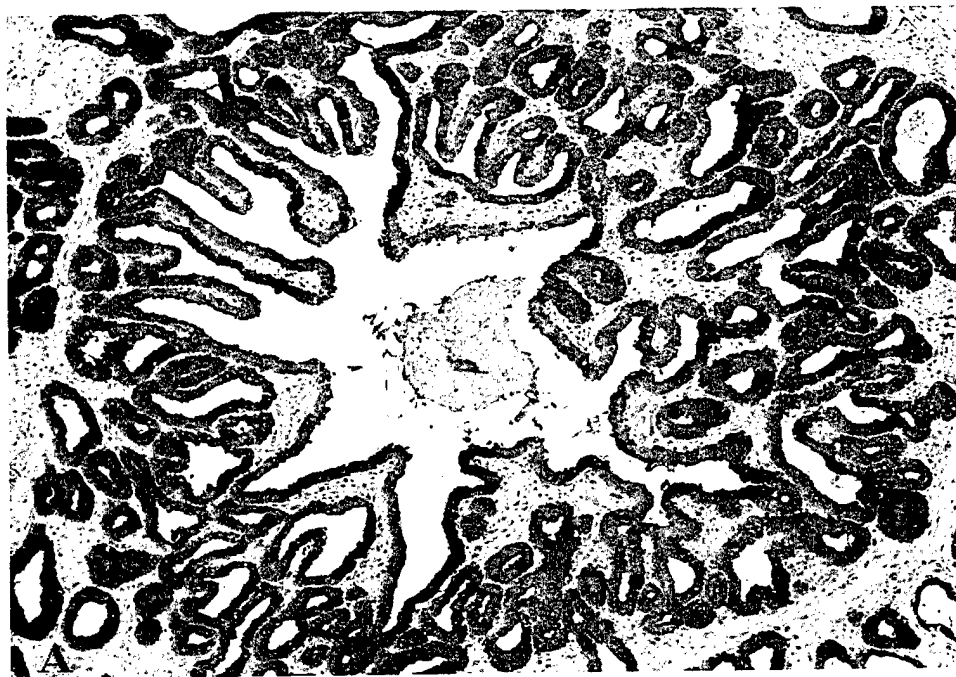
Figure 6: Panel **A** illustrates a normal seminal vesicle showing intense, diffuse positivity for the GST-π protein (100X). Panel **B** illustrates complete loss of GST-π protein expression in infiltrating prostatic adenocarcinoma (Gleason grade 6/10) adjacent to a portion of strongly positive seminal vesicle (100X). (anti-human GST–π, DAB, hematoxylin)
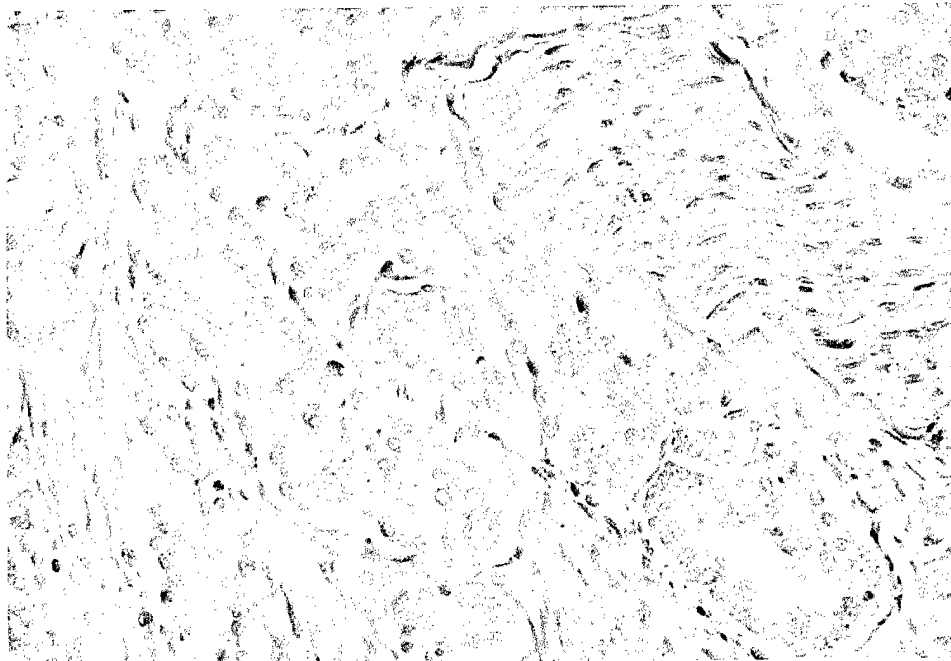
Figure 7: Example of a nerve showing immunoreactivity for the GST-π protein in comparison to the infiltrating prostatic adenocarcinoma which is negative for this protein (200X). (anti-human GST-π, DAB, hematoxylin)
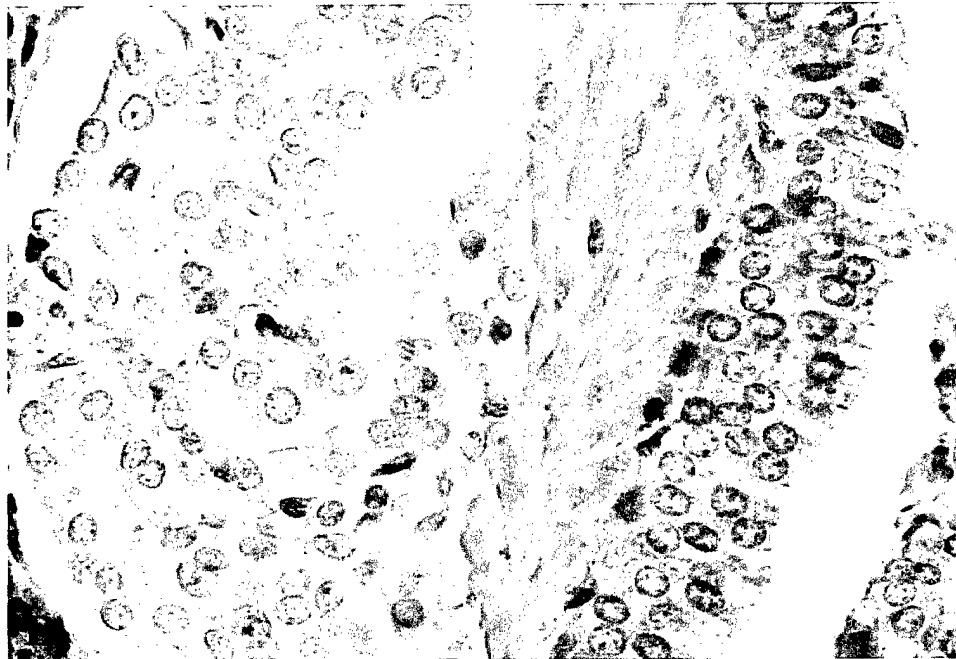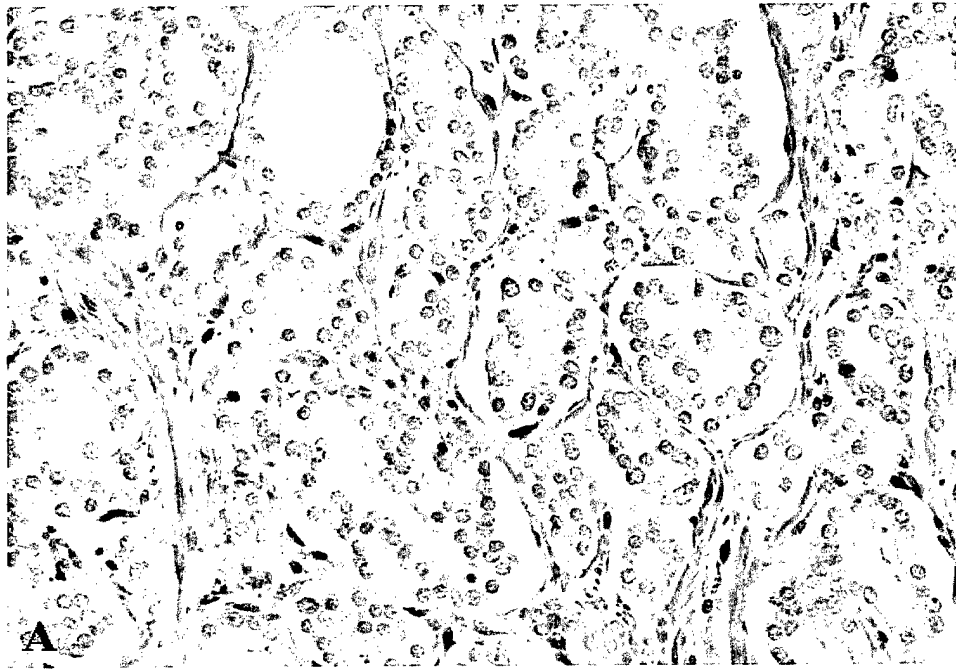
Figure 8: Panels **A** (200X) & **B** (400X) An example of low grade prostatic adenocarcinoma showing absence of GST-π protein expression. Note in Panel **B**, adjacent benign acinus is positive for this protein. (anti-human GST–π, DAB, hematoxylin)
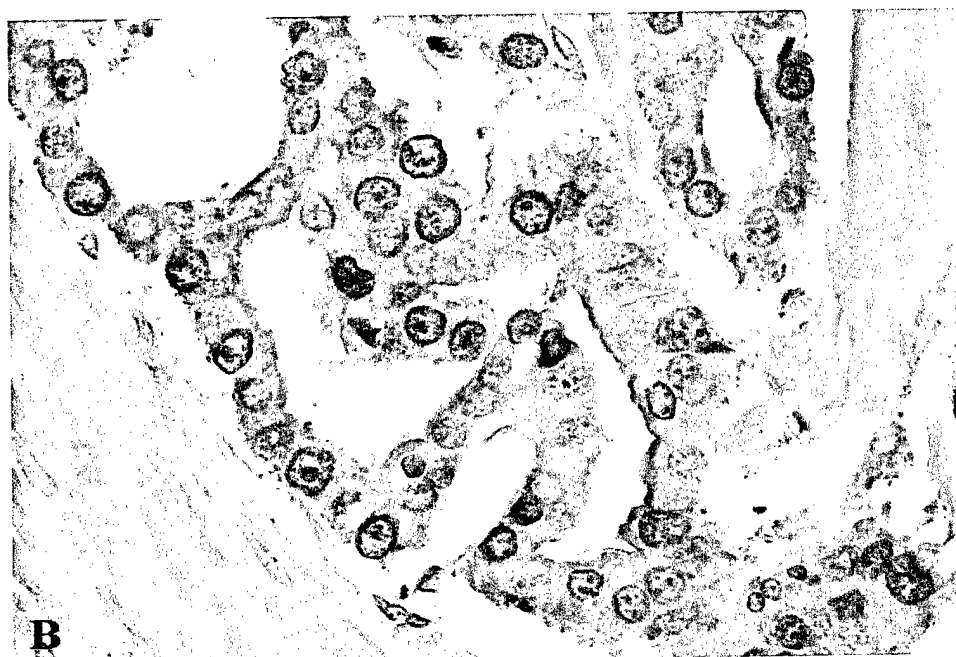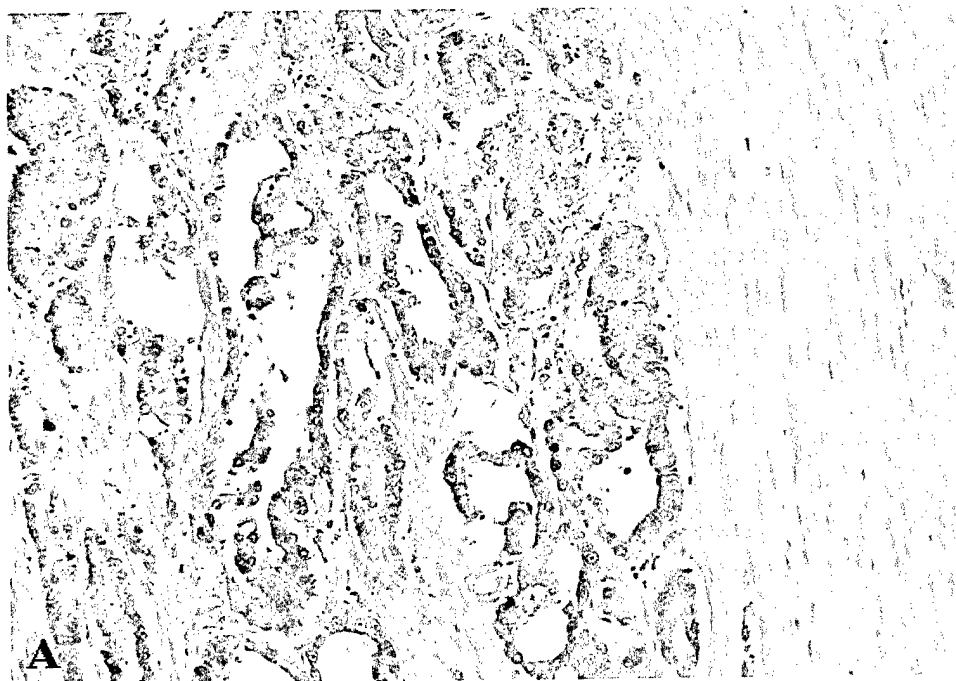
Figure 9: Panels **A** (200X) & **B** (400X) A rare example of a prostatic adenocarcinoma showing intense, diffuse, predominately cytoplasmic positivity for the GST-π protein. (anti-human GST-π, DAB, hematoxylin)
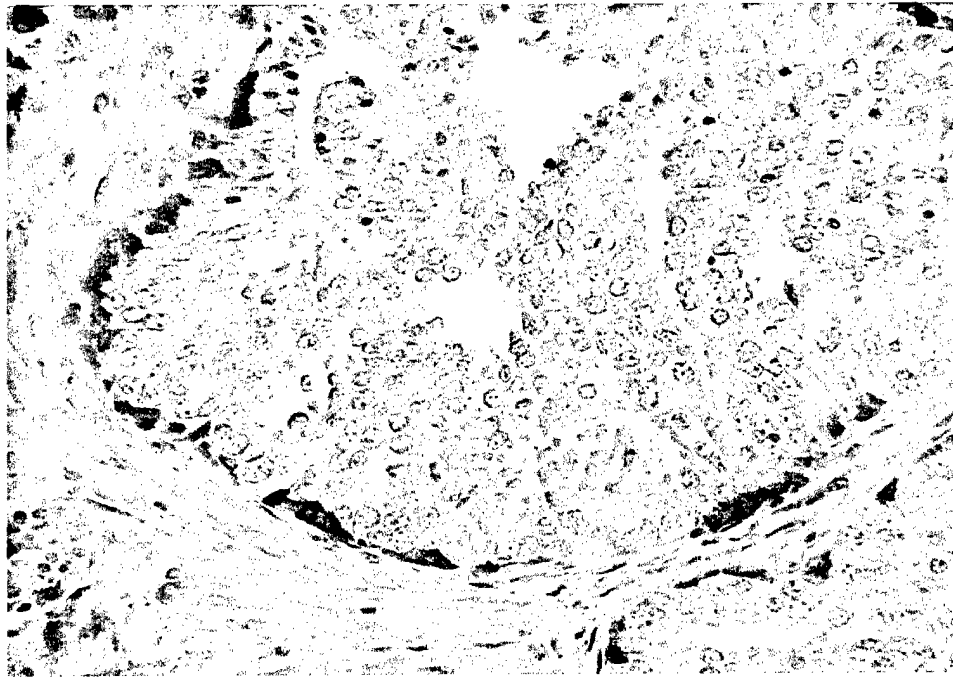
Figure 10: High grade PIN showing absence of expression of the GST-π protein. Note the intense positivity of the basal cells for this protein (200X). (anti-human GST–π, DAB, hematoxylin)

IMMUNOHISTOCHEMICAL DOWNREGULATION OF GST-π PROTEIN AND METHYLATION OF THE GENE PROMOTER ARE ASSOCIATED WITH MAJORITY OF PROSTATIC ADENOCARCINOMAS (PACs)

JS Ross, BVS Kallakury, CE Sheehan, SL Salmon, RD Anand, HAG Fisher, RP Kaufman Jr., CV Lowry.

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ABSTRACT

Glutathione-s-transferases (GSTs) are a family of enzymes that catalyze intracellular detoxification of carcinogens, the π family being the most widely studied isoenzyme. Recent studies have reported an association between loss of GST-π expression and abnormal patterns of DNA methylation in several cancers including human PACs. Formalin-fixed paraffin-embedded (FFPE) sections from 134 PACs were immunostained by an automated method (Ventana) using a polyclonal goat anti-human GST-π antibody (Novocastra). Results were semiquantitatively scored based upon both staining intensity and distribution. DNA was extracted (ex-wax™) from FFPE sections in 125 cases and subjected to sodium bisulfite modification. Following PCR amplification using primer pairs against methylated and unmethylated GST-π gene, the products were electrophoresed in a 4% NuSieve gel, stained with SYBR green and visualized with a STORM 860 Phospholmager (Molecular Dynamics), 119/134 (89%) PACs demonstrated a complete loss of GST-π protein which correlated with high tumor grade [58/60(97%)] high grade vs 61/74(82%) low grade, p=0.009]. 103/125(82%) PACs and 1/16(6%) benign tissues demonstrated methylated GST-π promoter DNA. There was no correlation between GST-π methylation and prognostic variables. In conclusion, a vast majority of PACs demonstrate both loss of immunohistochemical expression of GST-π protein and methylation of the gene promoter. Given its wide prevalence in PACs, GST-π methylation is not a prognostic marker but may serve as a valuable aid in the screening and diagnosis of prostate cancer.

INTRODUCTION

- Glutathione-s-transferases (GSTs) are a family of detoxification enzymes that provide protection to mammalian cells against a variety of toxins and carcinogens.

- Recent literature suggests that methylation of the GST-π gene, which inactivates the expression of the enzyme plays a role in prostate cancer evolution and spread and can be a cancer specific marker.

- The aim of this study is to evaluate GST-π protein expression by immunohistochemistry and develop a simple polymerase chain reaction assay for the detection of GST-π gene methylation status.

MATERIALS AND METHODS

- Formalin-fixed paraffin-embedded (FFPE) sections from 134 PACs were immunostained by an automated method (Ventana Medical Systems, Tucson, AZ) using a polyclonal rabbit anti-human GST-π antibody (Novocastra, Newcastle, UK).

- Results were semiquantitatively graded based upon staining intensity and distribution and correlated with histologic and prognostic parameters.

- DNA was extracted from FFPE sections of 125 PACs and 16 benign tissues, subjected to sodium bisulfite modification and analyzed by a gel based single primer pair PCR amplification method to evaluate for methylation status of the gene promoter.

- PCR primers for GST-π assay [annealing temperature 63°C]

5'CGTTTTTTGCGGTCGACGTTCG3/5'GCCAAGCGGCAAAAGCCCTTACCCCAAATC3'

5'GGATTTTTTAGAAGAGTGGTTGGTGTTCG3/5'ACAACCGACCAAACAAAAAACCCCTACCC3'

RESULTS

- While 100% of the admixed benign glandular epithelium expressed GST-π protein [Panel A], 119/134 (89%) PACs demonstrated a complete loss of this protein [Panel B] which correlated with high tumor grade [58/60(97%)] high grade [Panel B] vs 61/74(82%) low grade [Panel C]; p=0.009].

- Basal cells of benign prostatic acini [Panel C] demonstrated intense diffuse staining, in contrast to the loss of GST-π protein in foci of high grade PIN [Panel D].

- 103/125 (82%) PACs and 1/16 (6%) benign tissues demonstrated methylated GST-π promoter DNA [Panel E].

- There was no correlation between GST-π methylation status and prognostic variables.

PCR analysis of 8 PACs and 6 benign tissues using methylated (*) and unmethylated primers

CONCLUSIONS

- GST-π protein is downregulated in high grade PIN and a vast majority of prostatic carcinomas and is associated with methylation of the gene promoter.

- Further studies are warranted to evaluate the sensitivity and specificity of GST-π methylation assay in the screening and diagnosis of prostate cancer.

GST-π PROTEIN IS DOWNREGULATED IN HIGH GRADE PIN AND PROSTATIC ADENOCARCINOMAS (PACs): A PRELIMINARY ANALYSIS OF ASSOCIATION WITH METHYLATION OF THE GENE PROMOTER

BVS Kallakury, CE Sheehan, CV Lowry, SL Salmon, RD Anand, HAG Fisher, RP Kaufman Jr, JS Ross

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ABSTRACT

Background: Glutathione-s-transferases (GSTs) comprise a family of detoxification enzymes that provide protection to mammalian cells against a variety of toxins and carcinogens. Recent literature reported that methylation of the GST-π gene, which inactivates the expression of the enzyme, is a cancer specific marker in prostate tissues.

Design: Formalin-fixed paraffin-embedded (FFPE) sections from 134 PACs were immunostained by an automated method (Ventana Medical Systems, Tucson, AZ) using a polyclonal rabbit anti-human GST-π antibody (Novocastra, Newcastle, UK). Results were semiquantitatively graded based upon staining intensity and distribution and correlated with histologic and prognostic parameters. DNA was extracted from FFPE sections of 18 PACs and 6 benign tissues, subjected to sodium bisulfite modification and analyzed by a gel based single primer pair PCR amplification method to evaluate for methylation status of the gene promoter.

Results: 119/134 (89%) PACs demonstrated a complete loss of GST-π protein expression in comparison to its presence in 100% of admixed benign glandular epithelium. While basal cells of benign prostatic acini demonstrated intense diffuse staining, foci of high grade PIN demonstrated loss of GST-π protein. 58/60 (97%) high grade PACs demonstrated GST-π protein downregulation in comparison to 61/74 (82%) low grade tumors (p=0.009). 17/18 (94%) of PACs and 0/6 (0%) benign tissues demonstrated methylated GST-π promoter DNA.

Conclusions: GST-π protein is a marker of basal cell phenotype in prostate tissue, is downregulated in high grade PIN and carcinomas and is associated with methylation of the gene promoter. Further studies are warranted to evaluate the utility of GST-π methylation assay in the screening and diagnosis of prostate cancer.
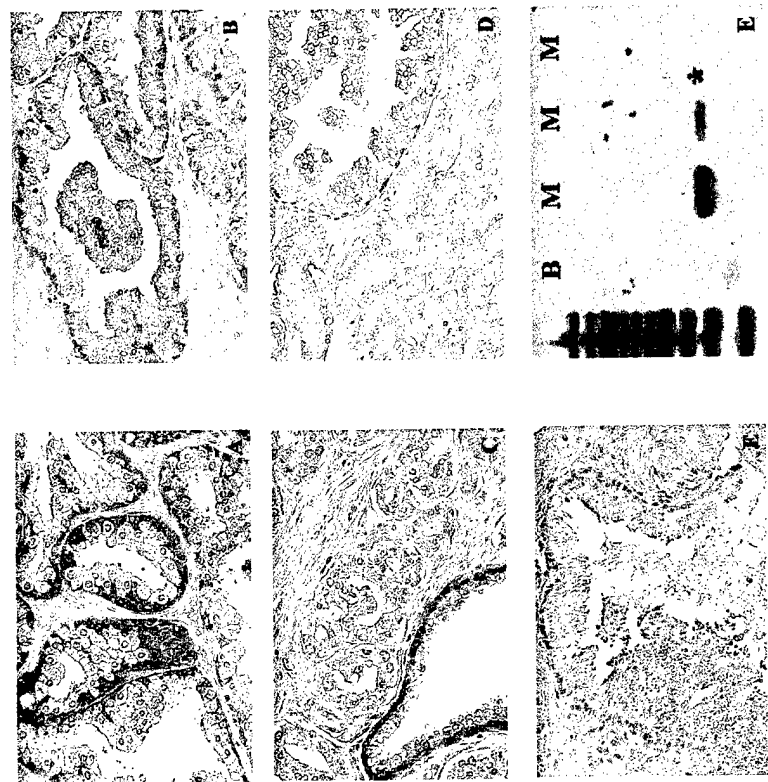
INTRODUCTION

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IMAGES

RESULTS

- 119/134 (89%) PACs demonstrated a complete loss of GST-π protein in comparison to its presence in 100% of admixed benign glandular epithelium [Panel B].

- While basal cells of benign prostatic acini demonstrated intense diffuse staining [Panel A], foci of high grade PIN demonstrated loss of GST-π protein [Panel E].

- 58/60 (97%) high grade PACs demonstrated GST-π protein downregulation [Panel D] in comparison to 61/74 (82%) low grade tumors (p=0.009) [Panel C].

- 17/18 (94%) of PACs and 0/6 (0%) benign tissues demonstrated methylated GST-π promoter DNA [Panel F].

CONCLUSIONS

- GST-π protein is a marker of basal cell phenotype in prostate tissue, is downregulated in high grade PIN and carcinomas and is associated with methylation of the gene promoter.

- Further studies are warranted to evaluate the utility of GST-π methylation assay in the screening and diagnosis of prostate cancer.